

Positive crosstalk between arginase-II and S6K1 in vascular endothelial inflammation and aging

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Summary

Augmented activities of both arginase and S6K1 are involved in endothelial dysfunction in aging. This study was to investigate whether or not there is a crosstalk between arginase and S6K1 in endothelial inflammation and aging in senescent human umbilical vein endothelial cells and in aging mouse models. We show increased arginase-II (Arg-II) expression/activity in senescent endothelial cells. Silencing Arg-II in senescent cells suppresses eNOS-uncoupling, several senescence markers such as senescence-associated- β -galactosidase activity, p53-S15, p21, and expression of vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1). Conversely, overexpressing Arg-II in non-senescent cells promotes eNOS-uncoupling, endothelial senescence, and enhances VCAM1/ICAM1 levels and monocyte adhesion, which are inhibited by co-expressing superoxide dismutase-1. Moreover, overexpressing S6K1 in non-senescent cells increases, whereas silencing S6K1 in senescent cells decreases Arg-II gene expression/activity through regulation of Arg-II mRNA stability. Furthermore, S6K1 overexpression exerts the same effects as Arg-II on endothelial senescence and inflammation responses, which are prevented by silencing Arg-II, demonstrating a role of Arg-II as the mediator of S6K1-induced endothelial aging. Interestingly, mice that are deficient in Arg-II gene (Arg-II^{-/-}) are not only protected from age-associated increase in Arg-II, VCAM1/ICAM1, aging markers, and eNOS-uncoupling in the aortas but also reveal a decrease in S6K1 activity. Similarly, silencing Arg-II in senescent cells decreases S6K1 activity, demonstrating that Arg-II also stimulates S6K1 in aging. Our study reveals a novel mechanism of mutual positive regulation between S6K1 and Arg-II in endothelial inflammation and aging. Targeting S6K1 and/or Arg-II may decelerate vascular aging and age-associated cardiovascular disease development.

Key words: aging; cellular senescence; endothelial cell; inflammation – molecular biology of aging; oxidative stress; signaling.

Introduction

Aging is a dominant risk factor for cardiovascular diseases (Najjar *et al.*, 2005). Emerging evidence shows that endothelial senescence, an irreversible

proliferation arrest in response to various stressors, plays a role in vascular aging and age-associated vascular diseases (Minamino *et al.*, 2002; Brodsky *et al.*, 2004; Erusalimsky & Skene, 2009; Rajapakse *et al.*, 2011). Senescent or aging endothelial phenotypes are characterized by oxidative stress, impaired eNOS function, and enhanced inflammatory molecule expression such as vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) (Shelton *et al.*, 1999; Minamino *et al.*, 2002; Fleenor *et al.*, 2012). The increase in endothelial adhesion molecule expression leads to enhanced adhesion and transmigration of monocytes into vascular wall, a key event in initiation and progression of atherosclerosis facilitated in aging (Maier *et al.*, 1993; Kovacic *et al.*, 2011). Growing evidence demonstrates that eNOS-uncoupling is an important mechanism of oxidative stress and decreased NO bioavailability in endothelial senescence, vascular aging, and age-associated cardiovascular diseases (Brandes *et al.*, 2005; Lemarie *et al.*, 2011; Rajapakse *et al.*, 2011). In the process of eNOS-uncoupling, the transfer of electrons from reductase domain to oxygenase domain where L-arginine is oxidized to vasoprotective NO, is bypassed to oxygen, resulting in enhanced production of O₂⁻ and diminished generation of NO from eNOS (Forstermann & Sessa, 2012).

A variety of mechanisms is implicated in eNOS-uncoupling in aging and cardiovascular pathologies (Forstermann & Sessa, 2012), which has not been fully elucidated, yet. Augmented arginase activity in endothelial cells has been reported to cause eNOS-uncoupling through relative deficiency in the intracellular substrate L-arginine (Kim *et al.*, 2009). There are two isoforms of arginase, i.e., Arg-I and Arg-II encoded by different genes (Morris *et al.*, 1997). Previous studies including our own showed that in human and murine endothelial cells, Arg-II is the predominant isoenzyme (Ming *et al.*, 2004; Scalera *et al.*, 2009) and inhibition of Arg-II improves endothelial function in animal models of atherosclerosis, diabetes, and aging (Ming *et al.*, 2004; Romero *et al.*, 2008; Kim *et al.*, 2009). However, a causal role of Arg-II in endothelial inflammatory responses and endothelial aging process has not been demonstrated, yet.

mTOR/S6K1, an evolutionarily conserved signaling pathway involved in regulation of a plethora of cellular functions (Selman *et al.*, 2009), is an important regulator of organism aging (Selman *et al.*, 2009; Ming *et al.*, 2012). Recent studies show that inhibition of S6K1 in mice extends lifespan (Chen *et al.*, 2009; Harrison *et al.*, 2009; Selman *et al.*, 2009). Our recent study demonstrates that persistent hyperactive S6K1 promotes endothelial senescence and eNOS-uncoupling in cultured human endothelial cells and in aortas of aged rats (Rajapakse *et al.*, 2011).

Taken into account that enhanced S6K1 and Arg-II activity both are involved in age-associated endothelial dysfunction, the present study is designed to investigate the interplay of S6K1 and Arg-II in promoting human endothelial aging and age-associated inflammatory responses in cell culture and mouse models.

Results

Senescent endothelial cells reveal enhanced expression of adhesion molecules and Arg-II

Our previous study showed that eNOS-uncoupling is an important mechanism of oxidative stress in senescent endothelial cells and vascular aging (Rajapakse *et al.*, 2011). Here we show that the senescent human

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endothelial cells, as confirmed by higher number of cells positively stained for SA- β -gal (Fig. S1A) and higher levels of other markers such as phosphorylated p53 at serine 15 (p53-S15) and the CDK inhibitor p21^{Cip1} (Pascos *et al.*, 2010) (Fig. S1B), express increased levels of VCAM1 and ICAM1 as compared to nonsenescent cells, referred to as 'young' cells. Moreover, the senescent endothelial cells display higher Arg-II protein levels with higher arginase activities than the young cells (Fig. S1C). Arg-I expression, however, could not be detected in the human endothelial cells.

Silencing Arg-II in senescent endothelial cells improves endothelial function and decreases adhesion molecule expression

We further show that silencing Arg-II in senescent endothelial cells, as validated by immunoblotting (Fig. 1A), significantly reduces O₂⁻ generation

(DHE staining) and enhances NO production (DAF-2DA staining) (Fig. 1B), reduces the number of SA- β -gal positive cells (Fig. 1C), and decreases levels of p21^{Cip1}, however, without affecting p53-S15 (Fig. 1D). In addition, silencing Arg-II in senescent cells significantly reduces VCAM1 and ICAM1 expression (Fig. 1D), demonstrating the critical role of Arg-II in endothelial inflammation and senescence.

Overexpressing Arg-II in young endothelial cells promotes endothelial dysfunction and adhesion molecule expression

Conversely, in nonsenescent cells, adenovirus-mediated ectopic expression of a wild-type (WT) Arg-II cDNA as verified by immunoblotting (Fig. S2A), leads to eNOS-uncoupling, i.e., impaired NO production (DAF-2DA staining) and enhanced intracellular O₂⁻ generation (DHE staining), which is significantly inhibited by the eNOS inhibitor L-NAME (1 mM, 1 h,

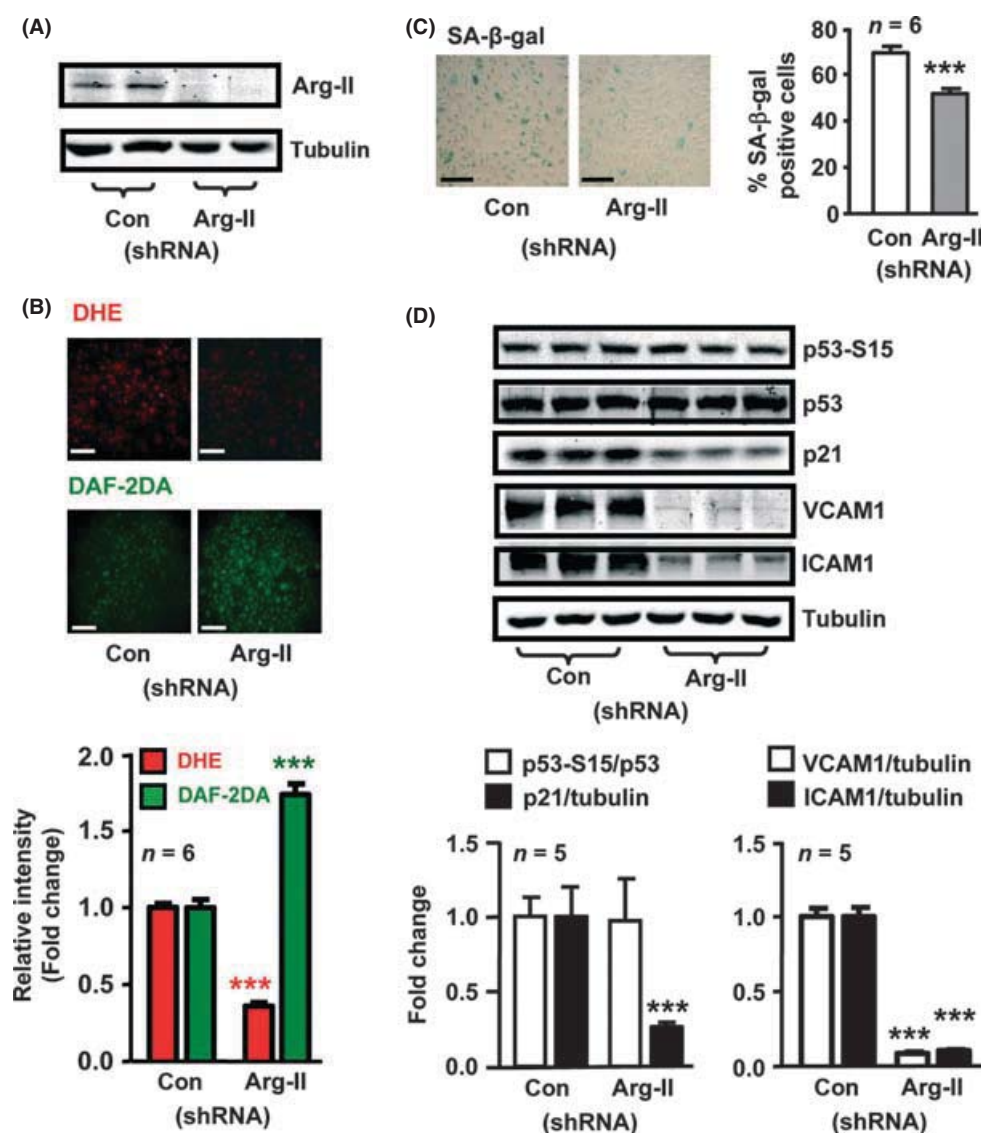


Fig. 1 Silencing Arg-II in senescent cells inhibits eNOS uncoupling, reverses endothelial senescent phenotypic changes, and suppresses endothelial inflammation. Senescent human umbilical vein endothelial cells (HUVECs) were transduced with rAd/U6-LacZ^{shRNA} as control (con) or rAd/U6-Arg-II^{shRNA} to silence Arg-II gene. (A) Immunoblotting shows Arg-II silencing in senescent cells. (B) DHE staining for detection of O₂⁻ and DAF-2DA staining for detection of NO. Quantifications of DHE and DAF-2DA signals are shown below. (C) SA- β -gal staining. Bar graphs show quantifications of SA- β -gal-positive cells. (D) Immunoblotting analysis of senescence markers p53-S15, p53, and p21^{Cip1} levels, and endothelial inflammation markers vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) expression. Bar graphs show quantifications of the markers. Tubulin served as protein loading control. ***P < 0.005 vs. control group (con). Scale bar = 0.2 mm.

Fig. S2B). Moreover, the senescence markers such as the number of SA- β -gal positive cells (Fig. S2C), the levels of p53-S15 and p21^{Cip1} as well as levels of VCAM1 and ICAM1 are significantly augmented by Arg-II overexpression (Fig. S2D). In contrast to the WT Arg-II, overexpression of an inactive Arg-II mutant in the young endothelial cells (Fig. S3A) does not induce eNOS-uncoupling (Fig. S3B), has no effects on SA- β -gal expression (Fig. S3C), the levels of p21^{Cip1}, VCAM1, and ICAM1 (Fig. S3D), although it seems to increase p53-S15 level. These results provide evidence for a causal role of Arg-II in promoting endothelial senescence and senescence-associated eNOS-uncoupling and inflammation, which is dependent on its enzymatic activity.

Recoupling of eNOS prevents Arg-II-induced endothelial inflammation and senescence

Since endothelial NO bioavailability has been shown to inhibit cell senescence and adhesion molecule expression (Khan *et al.*, 1996; Vasa *et al.*, 2000; Lemarie *et al.*, 2011), we further investigated if recoupling eNOS function is able to prevent senescence-promoting effects of Arg-II in the following experiments. For this purpose, superoxide dismutase-1 (SOD1) was co-expressed with Arg-II in young endothelial cells (Fig. 2A). We observe that eNOS-uncoupling evoked by Arg-II overexpression is prevented by co-expression of SOD1 (Fig. 2B), i.e., inhibition of O₂⁻ generation (DHE) and enhanced bioavailability of NO (DAF-2DA), demonstrating re-coupling of eNOS by SOD1. Cellular senescence and inflammatory responses caused by Arg-II gene overexpression, i.e., increased number of positively stained cells for SA- β -gal (Fig. 2C), elevated p53-S15 and p21^{Cip1} protein levels, and enhanced VCAM1 and ICAM1 expression (Fig. 2D) are all prevented by co-expressing SOD1. Accordingly, adhesion of THP-1 monocytes on endothelial cells is significantly enhanced in endothelial cells with Arg-II gene overexpression, which is inhibited by co-expression of SOD1 (Fig. 2E). These results demonstrate that eNOS-uncoupling is not only associated with endothelial senescence, but also mediates endothelial senescence and senescence-associated inflammation caused by Arg-II.

S6K1 up-regulates Arg-II gene expression

We recently showed that persistent hyperactive S6K1 promotes eNOS-uncoupling and endothelial senescence (Rajapakse *et al.*, 2011), an effect shared by Arg-II as demonstrated in the present study. These prompted us to investigate the role of S6K1 in regulation of Arg-II expression and activity. Indeed, in young endothelial cells, overexpression of a constitutively active S6K1 mutant (HA-S6K1ca), but not the inactive S6K1 mutant, which is confirmed by immunoblotting with the anti-S6K1 antibody that detects both mutants (Fig. 3A), enhances Arg-II mRNA and protein levels paralleled with increased arginase activity (Fig. 3A). Further experiments show that the Arg-II mRNA decay is slowed down in cells overexpressing the active S6K1ca, but not in those expressing the inactive S6K1 mutant (Fig. 3B). Conversely, silencing S6K1 in senescent cells reduces Arg-II mRNA, protein levels, and enzymatic activity (Fig. 3C), which is associated with faster decay of the Arg-II mRNA in the cells (Fig. 3D). These results demonstrate a stabilizing effect of hyperactive S6K1 on Arg-II mRNA in human endothelial cells. In agreement with the results of the S6K1 silencing, treatment of the senescent endothelial cells with the mTORC1-S6K1 inhibitor rapamycin (20 ng mL⁻¹, 1 h) or with the polyphenol resveratrol (10 μ M, 1 h), which has been shown to inhibit mTORC1-S6K1 signaling in the cells (Rajapakse *et al.*, 2011), also reduces Arg-II gene expression and activity (Fig. 4A). Similar results are observed in old mouse aortas treated with the two compounds (Fig. 4B). This part

of the results demonstrates that S6K1 up-regulates Arg-II levels at least in part through stabilizing its mRNA. In contrast, Arg-I mRNA and protein could not be detected using immunoblotting or qRT-PCR in the same experimental settings.

S6K1 promotes endothelial senescence phenotypes through Arg-II

Further experiments show that overexpressing the S6K1ca in young endothelial cells causes eNOS-uncoupling as evidenced by increased O₂⁻ (DHE) and reduced NO generation (DAF-2DA) (Fig. 5A), and increases the number of positively stained SA- β -gal cells (Fig. 5B), elevates p53-S15 and p21^{Cip1} levels (Fig. 5C), and enhances VCAM1 and ICAM1 expression (Fig. 5C), demonstrating that persistent hyperactive S6K1 promotes endothelial senescence and inflammation. Importantly, all the effects exerted by S6K1ca in young cells, including eNOS-uncoupling, endothelial senescence and VCAM1 and ICAM1 expression are reduced or diminished by Arg-II silencing. The effect of S6K1ca on Arg-II is abolished by Arg-II silencing (Fig. 54). In accordance with the augmented VCAM1 and ICAM1 expression, S6K1ca overexpression in young cells enhances THP-1 monocyte adhesion, which is also prevented by Arg-II silencing in the endothelial cells (Fig. 5D). These results demonstrate that S6K1 promotes endothelial senescence and inflammation through up-regulating Arg-II.

Arg-II^{-/-} mice are protected from age-associated endothelial inflammation and oxidative stress

Our previous study showed that hyperactive S6K1 accelerates endothelial senescence and causes eNOS-uncoupling in vascular aging in animal models (Rajapakse *et al.*, 2011). Here, we further show a higher Arg-II gene expression in aortas of the old mice as compared to the young animals (Fig. 6A). *En face* immunofluorescence confocal microscopy demonstrates enhanced endothelial VCAM1 and ICAM1 expression in the old WT mice as compared to the young WT mice (Fig. 6B). In contrast, an increased adhesion molecule expression is not observed in the old Arg-II^{-/-} mice (Fig. 6B). These results are further confirmed by immunoblotting analyses in aortic lysates (Fig. 6C), which is in accordance with the findings in senescent endothelial cells. Furthermore, the aging-associated increases in p53-S15, p53, and p21^{Cip1} in the WT mice are inhibited in the age-matched Arg-II^{-/-} animals (Fig. 6C-E). Also, the increase in O₂⁻ production (DHE staining) and decrease in NO production (DAF-2DA staining) in the aortas of aged WT mice are prevented in Arg-II^{-/-} animals (Fig. 55). These results from the animal model provide *in vivo* evidence showing that targeting Arg-II protects against eNOS-uncoupling, vascular inflammation, and vascular aging phenotypic changes.

Interestingly, an age-associated increase in S6-S235/S236 (p-S6), total S6, and the p-S6/total S6 ratio in the WT mouse aortas are observed, which is inhibited in Arg-II^{-/-} animals (Fig. S6A). Furthermore, silencing Arg-II in senescent endothelial cells is able to reduce S6-S235/S236 (p-S6) levels (Fig. S6B). These results demonstrate a positive regulation of S6K1 pathway by Arg-II in aging (Fig. S6C).

Discussion

Endothelial senescence and aging phenotypes are characterized by enhanced expression of inflammatory adhesion molecules, decreased NO production and increased oxidative stress caused by eNOS-uncoupling, and increased expression of aging markers (Shelton *et al.*, 1999; Minamino *et al.*, 2002; Zou *et al.*, 2004; Miles *et al.*, 2008; Passos *et al.*, 2010; Fleenor *et al.*, 2012). The endothelial aging phenotypes have been

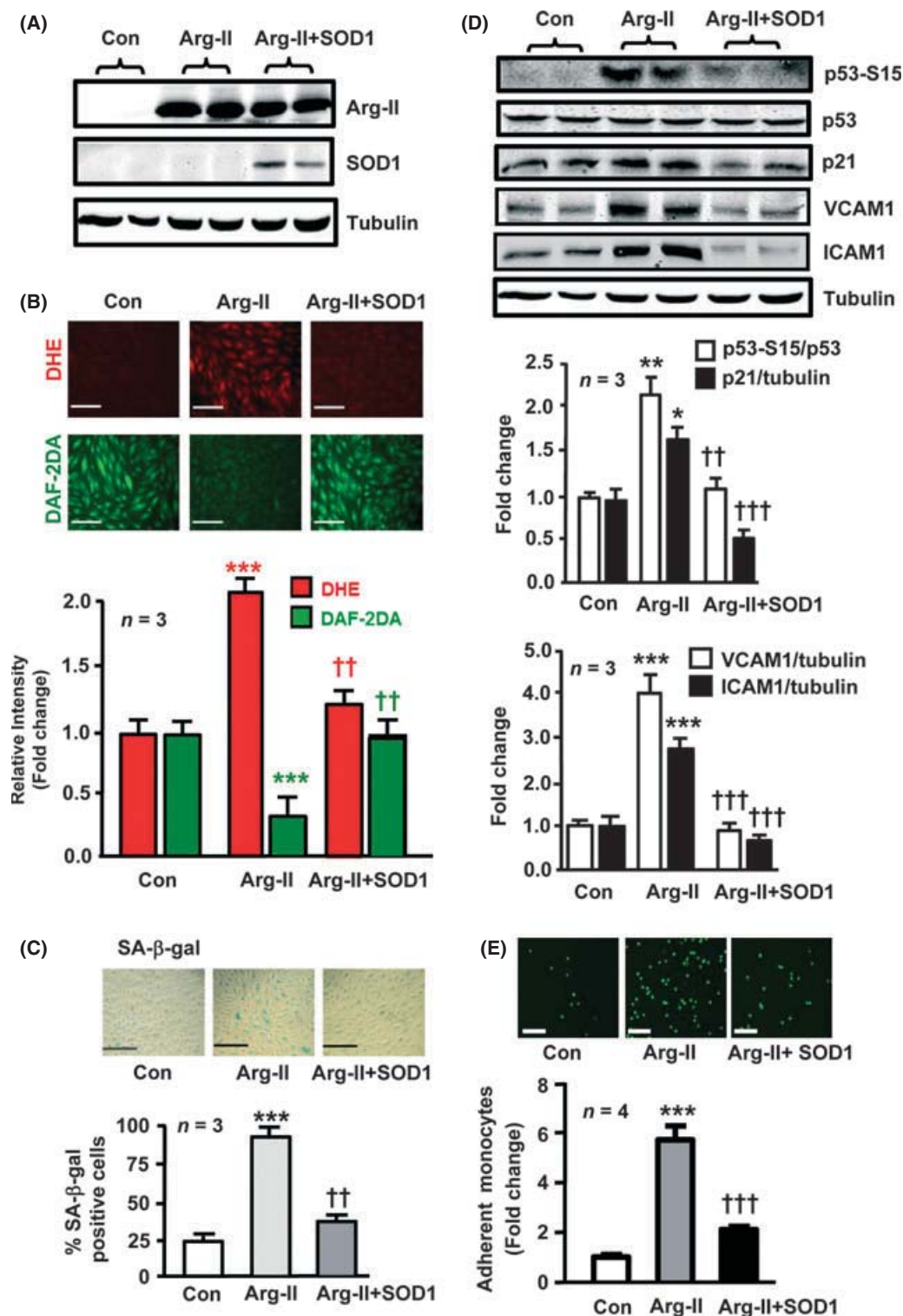


Fig. 2 Co-expression of superoxide dismutase-1 (SOD1) in young endothelial cells prevents Arg-11-induced eNOS-uncoupling, endothelial senescence and inflammation. Young endothelial cells were transfected with empty rAd/CMV vector as control (con) or rAd/CMV-Arg-11 alone or rAd/CMV-Arg-11 plus rAd/CMV-SOD1. (A) Immunoblotting analysis to confirm overexpression of Arg-11 and SOD1. (B) DHE staining for detection of O_2^- and DAF-2DA staining for detection of NO and effect of SOD1. Bar graphs show quantifications of DHE and DAF-2DA signals. (C) SA-β-gal staining and effect of SOD1. Bar graphs show quantifications of percentage of SA-β-gal positive cells. (D) Immunoblotting analysis of senescence markers p53-S15, p53, and p21^{CIP1} levels, and endothelial inflammation markers vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) expression. Tubulin served as loading control. Bar graphs show quantifications of the markers. (E) CFDA-SE fluorescence labeled THP-1 monocyte adhesion to endothelial cells that were transfected with rAd expressing transgenes as indicated. Bar graphs show quantifications of the adhered monocytes. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ vs. control (con); †† $P < 0.01$ and ††† $P < 0.005$ vs. Arg-11. Scale bar = 0.2 mm.

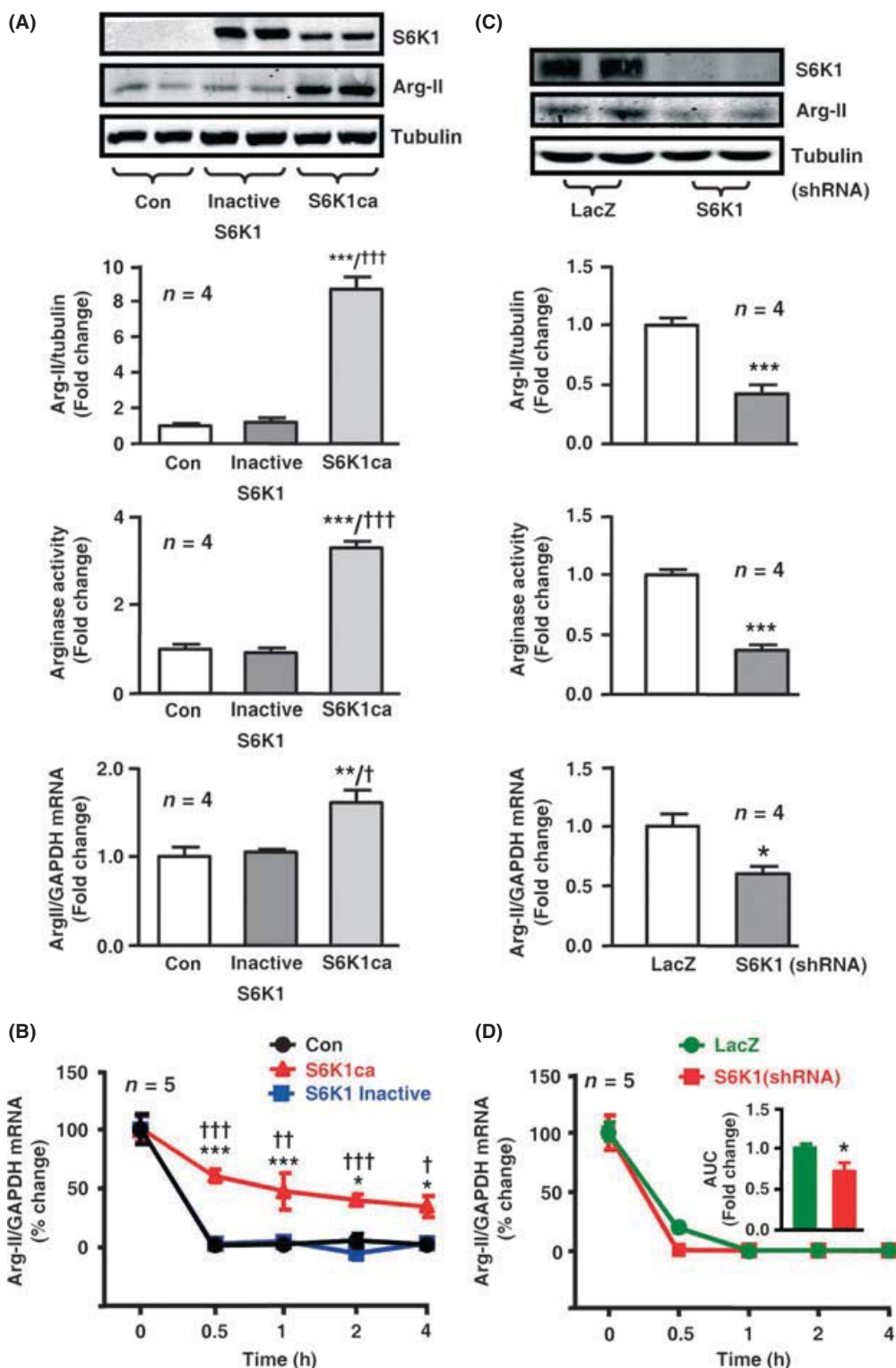


Fig. 3 S6K1 up-regulates Arg-II. (A) Effects of overexpression of the inactive S6K1 and the constitutively active S6K1ca mutants in young cells on Arg-II expression and activity. (B) Arg-II mRNA decay in young endothelial cells expressing empty vector serving as control (con), the inactive S6K1 mutant, and the active S6K1ca mutant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.005 vs. control (con); †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.005 vs. inactive S6K1. (C) Silencing S6K1 in senescent cells reduces Arg-II expression and activity. Senescent Human umbilical vein endothelial cells (HUVECs) were transfected with either rAd/U6-LacZ^{shRNA} as control or rAd/U6-S6K1^{shRNA}. Experiments were performed four days post transduction. Blots above reveal the immunoblotting analysis of S6K1 and Arg-II. Bar graphs below show quantifications of Arg-II/tubulin protein level, arginase activity, and Arg-II/GAPDH mRNA levels as analyzed by qRT-PCR. (D) Arg-II mRNA decay in senescent endothelial cells with S6K1 silencing. AUC = area under the curve; **P* < 0.05, ****P* < 0.005 vs. control (LacZ).

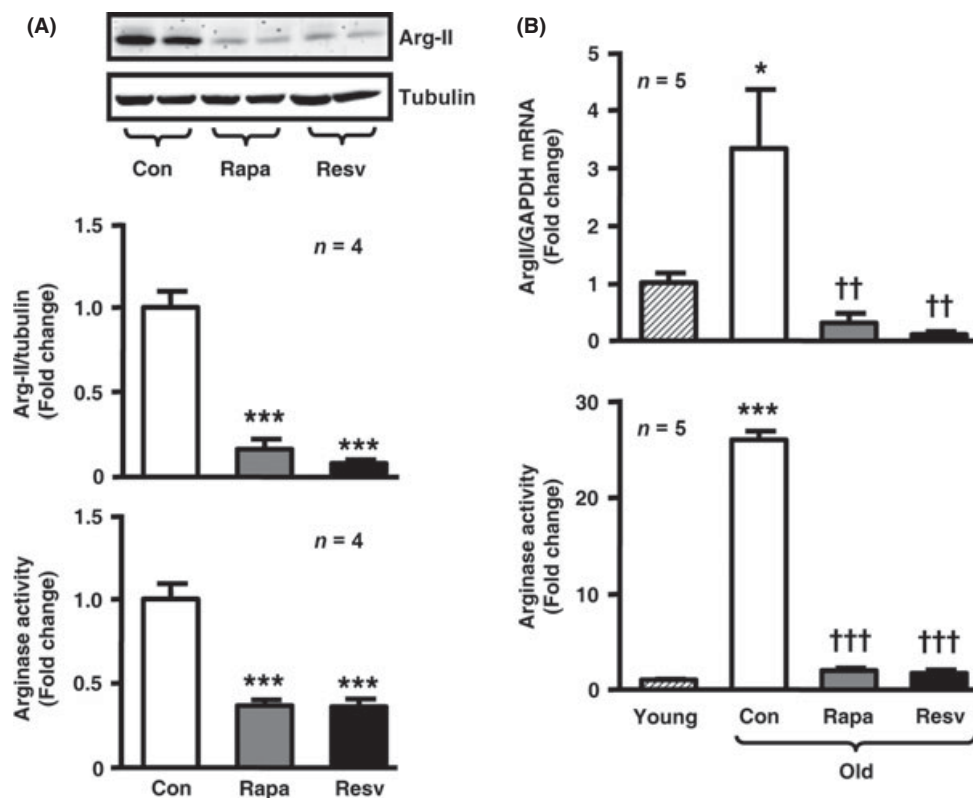


Fig. 4 Rapamycin and resveratrol inhibit Arg-II in aging. Treatment of senescent endothelial cells (A) or aortas of old wild-type mice (B) with rapamycin (Rapa, 20 ng mL⁻¹) or resveratrol (Resv, 10 μM) for 1 h decreases Arg-II expression analyzed either by immunoblotting or qRT-PCR, and arginase activity. **P* < 0.05, ****P* < 0.005 vs. control (Con, solvent) or young groups. ††*P* < 0.01, and †††*P* < 0.005 vs. old control (Con, solvent) groups.

considered to promote age-associated progression of atherosclerosis (Maier *et al.*, 1993; Kovacic *et al.*, 2011). How the endothelial senescence and aging phenotypes are regulated remains incompletely understood. Arginases including Arg-I and Arg-II have been shown to be involved in decreased eNOS functions under various pathological conditions and in aging (Ming *et al.*, 2004; Romero *et al.*, 2008; Kim *et al.*, 2009). Although Arg-I and Arg-II both are involved in eNOS dysfunction in rats (Kim *et al.*, 2009), Arg-II plays a predominant role in humans and mice (Ming *et al.*, 2004; Scalera *et al.*, 2009). Our current study further confirms these findings by showing that in the senescent human endothelial cells and aortas of aged WT mice, the Arg-II (but not Arg-I) gene expression is increased, which is paralleled with enhanced protein levels and higher arginase activities. We also provide additional evidence that Arg-II is not only involved in eNOS-uncoupling but also capable of promoting endothelial aging phenotypic changes including senescence markers and inflammatory adhesion molecule expression. Indeed, shRNA-mediated silencing of Arg-II in senescent human cells or genetic disruption of Arg-II in old mice preserves eNOS function, reduces adhesion molecule expression, resulting in decreased monocyte adhesion to the cells, and decreases endothelial senescence markers. Conversely, overexpression of the WT Arg-II gene in nonsenescent 'young' endothelial cells causes the senescent and aging phenotypic changes, whereas overexpression of the Arg-II inactive mutant has no effects on the aging parameters, except p53-S15. The results suggest that the effects of Arg-II on endothelial aging are dependent on its enzymatic activity, although some enzymatic activity-independent effect may also exist. It seems that regulation of p53 expression and p53-S15 by Arg-II in endothelial senescence and aging is complex. For example, Arg-II knockdown in senescent

cells has no effect on p53-S15 and p53 levels, whereas overexpression of Arg-II in nonsenescent cells increases the p53-S15 level. A possible explanation is that in aging, multiple redundant mechanisms are involved in enhanced p53-S15 and knockdown of Arg-II gene alone would not lower the p53-S15 level. Another discrepancy regarding p53 regulation is that in contrast to Arg-II knockdown in the senescent cells, knockout of Arg-II gene in mice (Arg-II^{-/-}) decreases total p53 protein levels. This might be explained by the different experimental conditions or models. Whether it is due to *in vitro* cell culture model vs. *in vivo* mouse model, or due to Arg-II knockdown in senescent cells (an experimental approach to reverse endothelial aging phenotype) vs. embryonic Arg-II knockout (an experimental approach to prevent vascular aging phenotype) remains to be investigated. Nevertheless, the results from the cultured cells and mouse model provide both *in vitro* and *in vivo* firm evidences for a causal role of Arg-II in promoting endothelial inflammation, eNOS dysfunction, and vascular aging.

Furthermore, we show that the promoting effects of Arg-II on endothelial aging and inflammation are mediated by eNOS-uncoupling. Previous work including that of ours demonstrated that vascular aging is associated with eNOS-uncoupling (Rajapakse *et al.*, 2011). Arginase has been shown to promote eNOS-uncoupling in aging animals (Kim *et al.*, 2009). In the current study, we show that silencing Arg-II in senescent cells recouples eNOS with concomitant reduction of cellular senescence markers and endothelial adhesion molecule expression. Further experiments show that abrogation of O₂⁻ by co-expression of SOD1 in nonsenescent cells over-expressing Arg-II not only recouples eNOS but also prevents Arg-II-induced senescence as well as senescence-associated endothelial adhesion molecule expression. The fact that Arg-II-induced

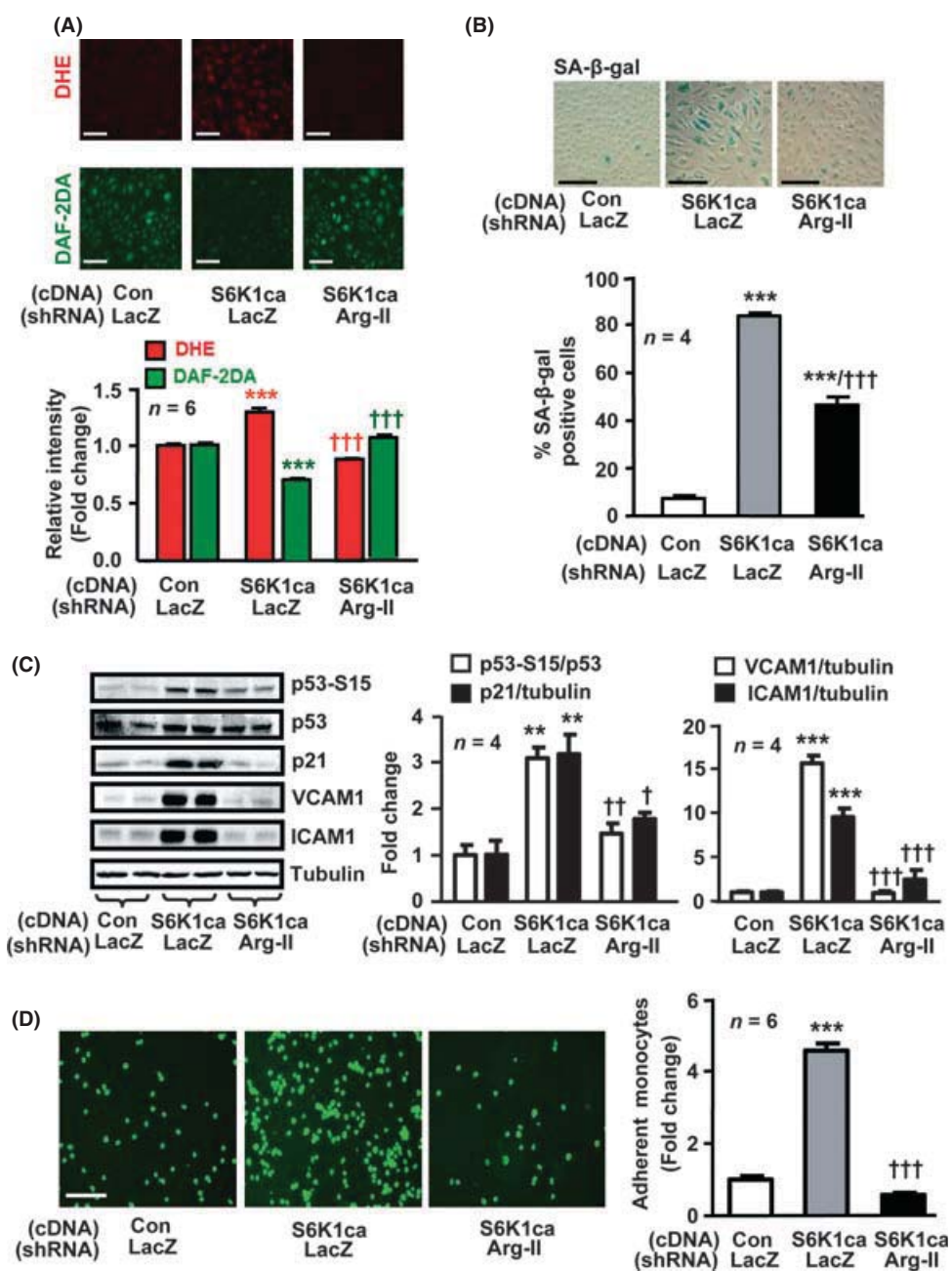


Fig. 5 Silencing Arg-11 prevents S6K1-induced eNOS-uncoupling, senescence and inflammation in young endothelial cells. The transduction procedure of young cells was the same as in Fig. 5A. (A) DHE staining for detection of O_2^- and DAF-2DA staining for detection of NO and effect of Arg-11 silencing. Bar graphs show quantifications of DHE and DAF-2DA signals. (B) SA-β-gal staining and effect of Arg-11 silencing. Bar graphs show quantifications of percentage of SA-β-gal positive cells. (C) Immunoblotting analysis of senescence markers p53-S15, p53, and p21^{Cip1} levels, and endothelial inflammation markers vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1). Tubulin served as loading control. Bar graphs show quantifications of the markers. (D) CFDA-SE fluorescence labeled THP-1 monocyte adhesion to endothelial cells that were transduced with rAd expressing transgenes and shRNA as indicated. Bar graphs show quantifications of the adhered monocytes. **P < 0.01, ***P < 0.005 vs. control (con/LacZ); †P < 0.05, ††P < 0.01, †††P < 0.005 vs. S6K1ca/LacZ group. Scale bar = 0.2 mm.

O_2^- production results from eNOS-uncoupling as evidenced by its L-NAME-inhibitable feature and that removal of Arg-11-induced O_2^- through co-expression of SOD1 enhances NO bioavailability suggests a positive regulatory circle between eNOS-uncoupling and oxidative stress. The O_2^- generated from initial eNOS-uncoupling is required to maintain the persistent eNOS-uncoupling that ultimately causes endothelial senescence and inflammation. These findings in cellular aging model are

further confirmed in Arg-11^{-/-} mice. Indeed, in the old Arg-11^{-/-} mice, the O_2^- generation from eNOS is decreased and NO production is preserved as compared to the WT mice, which is in line with the finding of Kim and colleagues who reported that chronic inhibition of arginase, most likely Arg-1 in old rats, leads to recoupling of eNOS, i.e., improved NO and reduced O_2^- generation from eNOS (Kim *et al.*, 2009). Importantly, the aging-associated increases in p21^{Cip1}, p53, and p53-S15, as well as

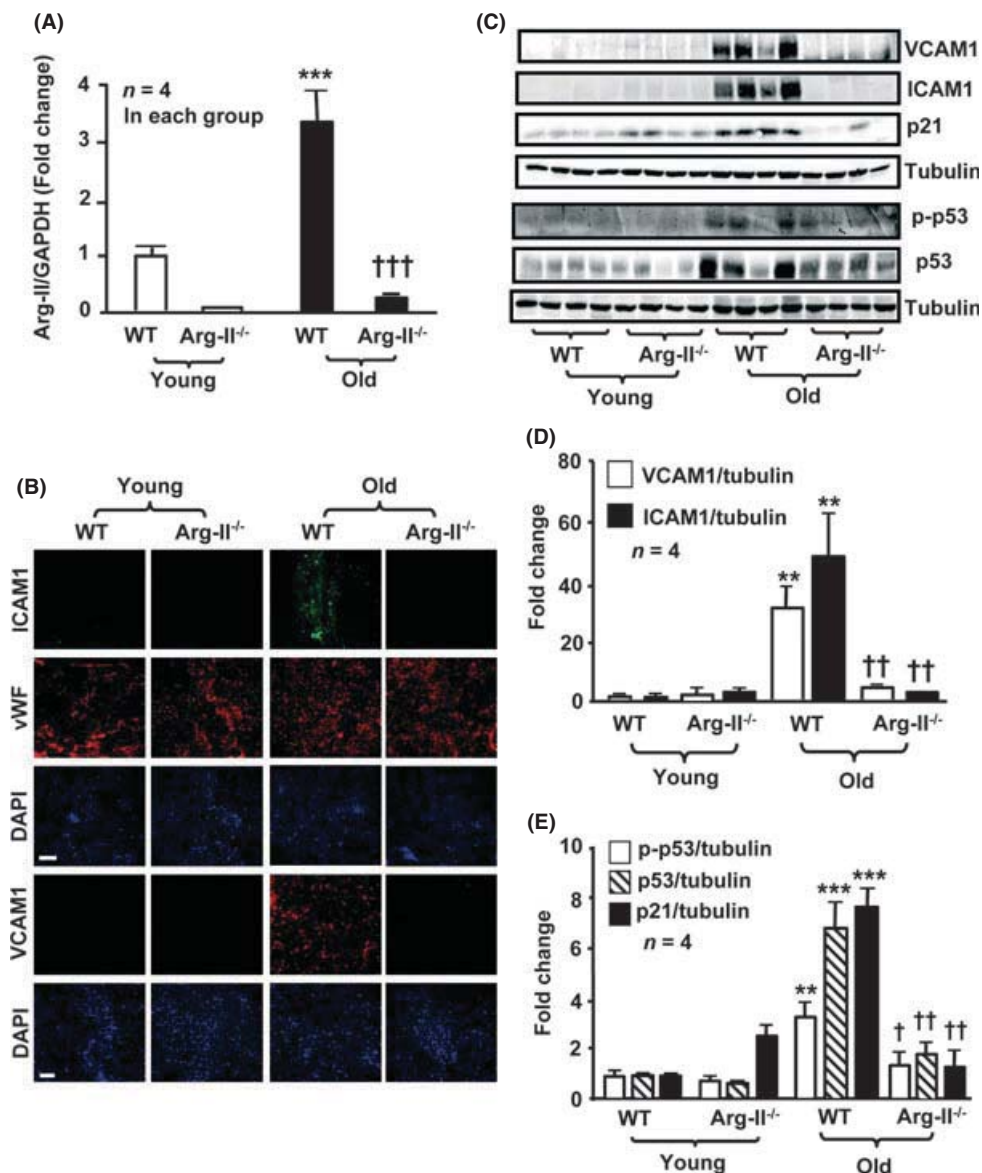


Fig. 6 Deficiency in Arg-II gene in mice (Arg-II^{-/-}) protects against vascular inflammation and aging. Aortas of young (2–3 months) and old (23–24 months) wild-type (WT) and Arg-II^{-/-} mice were cleaned of perivascular tissues and subjected to *en face* staining or Immunoblotting analysis. (A) qRT-PCR analysis of Arg-II mRNA levels. (B) Confocal microscopic *en face* detection of endothelial vascular adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1), vWF (the endothelial marker), followed by counterstaining with DAPI. Shown are representative images of each group. (C) Immunoblotting analyses of VCAM1, ICAM1, p21 levels in the aortas and p53-Ser15 and p53 levels in the heart of young and old WT and Arg-II^{-/-} mice. (D and E) Quantifications of the above results. Tubulin is taken as loading control. n = 4 mice in each group. **P < 0.01, ***P < 0.001 vs. young WT mice; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. old WT mice. Scale bar = 100 μ m.

adhesion molecule expression in the aortas are abrogated in the old Arg-II^{-/-} mice, demonstrating the causal role of Arg-II in cardiovascular aging *in vivo*.

We recently showed that S6K1 activity is persistently high in senescent endothelial cells and aortas of aged rodents, which promotes eNOS-uncoupling and endothelial senescence (Rajapakse *et al.*, 2011). Here, we further demonstrate that overexpression of a constitutively active S6K1 mutant, but not the inactive mutant, up-regulates Arg-II (not Arg-I) gene expression paralleled with increased arginase activity in nonsenescent cells. The fact that overexpression of the active S6K1 mutant in young endothelial cells enhances arginase activity with concomitant increase in Arg-II expression and that Arg-II^{shRNA} fully abolishes S6K1-induced Arg-II expression and decreases arginase activity to the basal level

of the control cells further demonstrates that S6K1-induced arginase activity is fully attributed to enhanced expression of Arg-II but not Arg-I. These results support the previous observation that Arg-II is the main isoform in human endothelial cells. Conversely, silencing S6K1 in senescent cells reduces Arg-II gene expression and activity (Ming *et al.*, 2004; Scaleria *et al.*, 2009). In line with these results, pharmacological inhibition of mTORC1-S6K1 by rapamycin or resveratrol that has been shown to inhibit S6K1 signaling (Rajapakse *et al.*, 2011) in senescent cells or old rat aortas decreases Arg-II gene expression and activity. The results for the first time demonstrate a critical role of hyperactive S6K1 in up-regulating Arg-II gene expression resulting in enhanced arginase activity in endothelial senescence. Importantly, we show that the promoting effects of S6K1 on eNOS-uncoupling, adhesion molecule expression, enhanced

monocyte-endothelial cell interaction, and cell senescence markers are inhibited by Arg-II silencing. These data thus provide firm evidences that S6K1 induces endothelial senescence and inflammation through up-regulating Arg-II mRNA levels.

Moreover, we demonstrate that the upregulation of Arg-II mRNA by S6K1 is at least in part through stabilizing its mRNA, since overexpression of active S6K1 in young cells slows down, whereas silencing S6K1 in senescent cells accelerates the Arg-II mRNA decay. The exact underlying mechanisms remain elusive. Sequence analysis of human Arg-II mRNA reveals the presence of adenine/uridine-rich elements (AREs) consisting of four AUUUA motifs in its 3' untranslated region (3' UTR), which are characteristic of short-lived mRNAs (Barreau *et al.*, 2006). The stability of ARE-containing mRNAs is regulated by AU-binding proteins (AUBPs) which can be modulated by the signal transduction pathways such as the mitogen-activated protein kinase (MAPK) and PI3K-Akt (Khabar, 2010). It is presumable that S6K1 may enhance the stability of Arg-II mRNA through modulation of AUBPs. This aspect warrants further investigation.

Another important novel finding of our study is that Arg-II also positively stimulates S6K1 in vascular aging. This is evidenced by the fact that silencing Arg-II gene in senescent endothelial cells inhibits S6K1 activity and Arg-II gene knockout in mouse abolishes age-associated hyperactive S6K1 in the aortas. Further work should investigate the molecular mechanisms of Arg-II-mediated S6K1 activation in the endothelial cells.

Taken together, our study provides the first evidence for a crosstalk between S6K1 and Arg-II in vascular endothelial aging (Fig. S6C). The mutual positive regulation between S6K1 and Arg-II gene expression accelerates endothelial aging through eNOS-uncoupling. The results suggest that interruption of S6K1-Arg-II crosstalk may represent a promising therapeutic strategy to decelerate vascular aging and age-associated cardiovascular diseases.

Experimental procedures

Materials

All chemicals including those used for immunoblotting and anti-tubulin (T5168) antibody were obtained from Sigma (Buchs, Switzerland). Antibody against p21^{Cip1} (OP64) was purchased from Calbiochem (Genève, Switzerland); antibody against phosphor-p53-S15 (#9284s) was from Cell Signalling (Allschwil, Switzerland); Antibodies against S6K1 (#9205s) were from BD Transduction laboratories (Allschwil, Switzerland); antibodies against arginase-II (sc-20151) and p53 (sc-6243) were from Santa-Cruz (Nunningen, Switzerland); anti-SOD1 (TA302692) was from OriGene Technologies, Inc (Nunningen, Switzerland); Monoclonal antibody against HA-tag (12CA5) was obtained from Dr Brian A. Hemmings (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland); Alexa Fluor680-conjugated anti-mouse IgG (A21057) and dihydroethidium (DHE) were from Molecular Probes/Invitrogen (Lucerne, Switzerland); IRDye800-conjugated anti-rabbit IgG (926-32211) were from LI-COR Biosciences (Bad Homburg, Germany); the membrane-permeable 4,5-diaminofluoresceine acetate (DAF-2DA) was from VWR international SA (Dietikon, Switzerland); X-gal was from Promega (Dübendorf, Switzerland); Endothelial cell growth supplement (ECGS) pack was from PromoCell GmbH (Allschwil, Switzerland) and all cell culture media and materials were purchased from Gibco BRL (Lucerne, Switzerland).

Generation of recombinant adenoviral (rAd)

Expression plasmids encoding a murine arginase-II (pCMV6-kan/neo-ArgII) and a murine superoxide dismutase-1 (SOD1) (pCMV6-kan/

neo-SOD1) were purchased from OriGene Technologies, Inc. An inactive mutant of murine Arg-II in which the histidine160 was mutated to phenylalanine (Arg-II-H160F) (Falk *et al.*, 2001; Mauricio *et al.*, 2001) was generated using Phusion[®] Site-Directed Mutagenesis Kit according to manufacturer's instruction (Finnzymes, Espoo, Finland). The expression plasmid encoding a myc-tagged inactive mutant of S6K1 (pRK5-myc-S6K1-Q100) was kindly provided by Dr George Thomas (Schwab *et al.*, 1999). Recombinant adenoviruses expressing wild-type Arg-II, Arg-II-H160F, SOD1, and myc-S6K1-Q100 were carried out with the Gateway Technology (Invitrogen life Technologies) according to manufacturer's instruction. rAd/CMV-HA-S6K1ca (a HA-tagged constitutively active S6K1 mutant HA-F5A-E389-R3A) and rAd expressing shRNA targeting human Arg-II and S6K1 driven by the U6 promoter (rAd/U6-hArg-II^{shRNA} and rAd/U6-hS6K1^{shRNA}) were described previously (Ming *et al.*, 2009, 2010). The control rAd expressing LacZ^{shRNA} and the empty rAd/CMV were from Invitrogen life Technologies.

Animals

The Arginase II-deficient mice (Arg-II^{-/-}) were kindly provided by Dr William O'Brien (Shi *et al.*, 2001) and backcrossed to C57BL/6J more than eight generations. Genotyping was performed by polymerase chain reaction (PCR) as previously described (Shi *et al.*, 2001). The WT and Arg-II^{-/-} offsprings from hetero/hetero cross were interbred to obtain WT and Arg-II^{-/-} mice, respectively, for experiments. Both WT and Arg-II^{-/-} mice were maintained on a 12-h light-dark cycle and fed standard chow diet and tap water according to the local guidelines of animal experimentation. The female young (2–3 months old) and old (23–24 months old) animals were anesthetized with xylazine (10 mg kg⁻¹ body weight, intraperitoneally) and ketamine (100 mg kg⁻¹ body weight, intraperitoneally) and sacrificed. Thoracic aortas were dissected and cleaned from perivascular fat, and subjected to *en face* staining or snap frozen directly in liquid nitrogen and kept at -80 °C till further biochemical analyses.

Endothelial cell culture and adenoviral transduction of the cells

Human umbilical vein endothelial cells (HUVEC) and senescent cells were prepared and transduction of HUVECs by recombinant adenovirus was performed as previously described (Rajapakse *et al.*, 2011). Briefly, cells of passage 1–2 (P1–P2) were used as young cells. For preparation of senescent cells, the cells were further split in a ratio of 1:3 continuously over a period of several weeks till replicative senescence was reached as assessed by SA-β-gal staining (P9–P12) (Rajapakse *et al.*, 2011). In the article, nonsenescent endothelial cells are referred to as 'young' cells.

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-galactosidase staining was performed 5 days post transduction as described (Rajapakse *et al.*, 2011).

Effects of rapamycin and resveratrol on Arg-II expression and activity in senescent endothelial cells and aged mouse aortas

Senescent endothelial cells in culture were treated either with rapamycin (20 ng mL⁻¹) or resveratrol (10 μM) or ethanol as solvent as control for 1 h. The cells were harvested either for immunoblotting analyses of Arg-II

protein levels or for arginase activity assay. Thoracic aortas from the young and old wild-type mice cleaned of perivascular tissues were cut into three segments, which were incubated either with rapamycin (20 ng mL⁻¹) or resveratrol (10 μM) or ethanol as solvent as control for 1 h in Krebs-Ringer bicarbonate solution at 37°C, aerated with 95% O₂/5% CO₂. The blood vessels were snap frozen and homogenized either for qRT-PCR analysis of Arg-II mRNA or for arginase activity measurement.

Arginase activity assay

Arginase activity was measured by colorimetric determination of urea formed from L-arginine as previously described (Ming *et al.*, 2004).

Immunoblotting

Cell lysate preparation, SDS-PAGE, and immunoblotting, antibody incubation and signal detection were performed as described (Rajapakse *et al.*, 2011). Quantification of the signals was performed using NIH IMAGE J (NIH, Bethesda, MD, USA).

Quantitative real-time reverse transcription PCR (qRT-PCR) analysis

Quantitative real-time reverse transcription PCR was performed as described previously (Ming *et al.*, 2010). mRNA expression of the endogenous Arg-II in HUVECs and in mouse aortas was evaluated by two-step qRT-PCR analysis. Total RNA was extracted from cells or aortas with Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the supplier's protocol. The mRNA expression levels of Arg-II were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences are as follows: hArg-II-F: ggctgaggtggttagcagag, hArg-II-R: ctggctgtccatggagatt; hArg-I-F: ggctgtctgtctgagaac, hArg-I-R: attgccaaactgtgtctcc; hGAPDH-F: tgaccaccaactgcttagc, hGAPDH-R: ggcattgactgtggtcatgag; mARGII-F: ccccttctctcggggacagaa, mARGII-R: gggcgtgaccgataatggt; mArg-I-F: ggaatctgcatgggcaacctgtgt, mArg-I-R: agggctacgtctcgaagcca; mGAPDH-F: acccagaagactgtggatgg, mGAPDH-R: acacattggggtaggaaca (h: human; m: mouse; F: forward primer; R: reverse primer).

Arg-II mRNA stability assay

Young HUVECs were transduced either with rAd/CMV vector as control (con) or rAd/CMV-S6K1 constitutive active or -S6K1 inactive mutant to over-express S6K1 mutants. Senescent HUVECs were transduced with rAd/U6-LacZ^{shRNA} as control (con) or rAd/U6-S6K1^{shRNA} to silence S6K1 gene. Two or 4 days post transduction with rAd/CMV-S6K1 or rAd/U6-S6K1^{shRNA}, respectively, actinomycin D (5 μg mL⁻¹) was added at time zero, total RNA was isolated at 30 min, 1, 2, and 4 h and subjected to qRT-PCR to determine Arg-II mRNA decay. The Arg-II mRNA levels were normalized to GAPDH. Percentage change to the Arg-II mRNA levels at time zero was presented.

Detection of NO and superoxide level in cultured endothelial cells and in intact mouse aortas

NO and superoxide levels in cultured endothelial cells as well as in intact mouse aortas were assessed by staining the cells or aortas *en face* with fluorescent dyes DAF-2DA and DHE, respectively, as described previously (Rajapakse *et al.*, 2011).

En face detection of VCAM1 and ICAM1 in intact mouse aortas

This experiment was performed as described (Nigro *et al.*, 2011). Young (2–3 months) and old mice (23–24 months) aortas of WT and Arg-II^{-/-} cleaned of perivascular tissues were fixed in 2% paraformaldehyde for 15 min followed by two washes in phosphate-buffered saline (PBS). Blocking was performed using 2% BSA in PBS for 1 h. The aortas were then incubated with primary antibody for VCAM1, ICAM1 and vWF at 4 °C overnight (all primary antibodies were diluted according to manufacturer's guidelines in 2% BSA in PBS). After washing three times in PBS, the aortas were further incubated with a corresponding goat anti-rabbit IgG(H+L) (Alexa Fluor-594 conjugated) or anti-mouse IgG (H+L) (Alexa Fluor-488 conjugated) for 1 h. The aortas were then washed three times followed by counterstaining with DAPI (300 nm, 3 min). After washing, the aortas were carefully cut longitudinally and mounted *en face* (face down) on slides and covered with cover slip for endothelial layer imaging. Vectashield mounting medium was used to preserve the fluorescence. The fluorescence was analyzed in a Leica DMI6000 confocal microscope within h after preparation. Fluorescence from ICAM1 staining was excited with 488 nm argon laser with emission detection at 500–535 nm, whereas fluorescence for VCAM1 and vWF was detected at 590 nm emission. Z-scanning was done for each sample. After the signal on the top (endothelial layer on the lumen border) of the sample was observed, the images were collected. Three consecutive images per field, acquired through the full thickness of endothelial signal, were recorded for analysis. At least three different fields per sample were evaluated. The images from VCAM1, ICAM1, vWF and DAPI staining were quantified with NIH IMAGE J and results are presented as the ratio of VCAM1, ICAM1 to DAPI positive nucleus. vWF is used to confirm presence of endothelial layer.

Monocyte adhesion to endothelial cells

The human monocytic cell line THP-1 was cultured in RPMI-1640 containing 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS). For adhesion assays, THP-1 cells were labeled with 5 μM CFDA-SE in PBS at 37 °C for 8 min. The labeling was stopped with 1 mL of heat-inactivated FBS for 1 min. The labeled monocytes (4 × 10⁵ THP-1) were then added to the HUVECs that were transduced with recombinant adenoviruses and serum-starved in medium containing 0.2% fetal calf serum (FCS) for 12 h prior to the addition of labeled monocytes. After incubation for 15 min at 37 °C, the nonadherent THP-1 cells were washed twice with PBS and fixed in 2% paraformaldehyde. The images of adherent monocytes were captured under the fluorescent microscope (three different fields per sample were captured). The number of adherent monocytes was counted using the NIH IMAGE J.

Statistics

Data are given as mean ± SEM. In all experiments, *n* represents the number of experiments or animals. Statistical analysis was performed with unpaired *t*-test or ANOVA with Bonferroni post test. Differences in mean values were considered significant at *P* < 0.05.

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Conflict of Interest

None.

Author contributions

Gautham Yepuri, Srividya Velagapudi, Yuyan Xiong, and Angana G. Rajapakse performed experiments, analyzed and interpreted data and approved the final submission; Jean-Pierre Montani, Xiu-Fen Ming and Zhihong Yang initiated and designed the project, drafted the manuscript for important intellectual content, interpreted the data and approved the final submission.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Senescent endothelial cells exhibit enhanced Arg-II expression and activity.

Fig. S2 Overexpression of Arg-II gene in young cells induces eNOS uncoupling, endothelial senescence, and inflammation.

Fig. S3 Enzymatic activity dependence of the Arg-II-promoted endothelial aging in young cells.

Fig. S4 Silencing Arg-II prevents S6K1-induced Arg-II expression and arginase activity.

Fig. S5 Deficiency in Arg-II gene in mice (Arg-II^{-/-}) improves eNOS function in aging.

Fig. S6 S6K1 activity in aging is positively regulated by Arg-II.

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